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PRINCIPAL INVESTIGATOR: Douglas S. Conklin, Ph.D.

CONTRACTING ORGANIZATION: The Research Foundation
                           State University of New York
                           Albany NY 12201-0009

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Douglas S. Conklin, Ph.D.

E-Mail: dconklin@albany.edu

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14. ABSTRACT We have identified approximately 30 genes that negatively impact the growth of Erb-B2 positive breast cancer cells. Year 2 of funding resulted in tests of the specificity of the shRNAs' negative impact on the growth of Erb-B2 positive breast cancer cells. Effects of these shRNAs on normal cells and other breast cancer cell lines, identified approximately 20 shRNAs that specifically inhibit Erb-B2 positive breast cancer cells target genes. Several of these gene targets are known to be important to a variety of cancers. The PBP and NR1D1 genes are interesting because they give new insight into a pathway that can be exploited as a potential therapeutic target. Year 3 of funding resulted in finding that inhibition of PBP-PPAR?-NR1D1 does not affect ERBB2 signaling in breast cancer cells. That this pathway is overactive in her-2/neu breast cancer cells is likely due to the tight genetic linkage of Nr1D1 and her-2. We have also found that the PBP-PPAR?-NR1D1 pathway alters fat metabolism in breast cancer cells and have identified lipotoxicity as a likely mechanism for triggering apoptosis in the her-2/neu positive breast cancer cells.

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Table of Contents

Introduction

The recent explosion of genome sequences and bioinformatic data has changed the nature of mammalian genetics. Systematic investigation of expression patterns of thousands of genes in breast tumors using cDNA microarrays and serial analysis of gene expression has yielded a long list of genes whose increased activity is correlated with the occurrence of breast cancer (1-4). In notable cases, associations between gene expression levels and clinical outcome have been determined. In most cases, however, the information is only correlative and serves as a rough guide to genes that might positively influence some aspect of breast cancer.

We are working towards identifying which of the nearly 100 genes typically overexpressed in ErbB-2 positive breast tumors are important for the survival and aggressiveness of breast cancer cells. Our approach makes use of DNA-encoded short hairpin RNAs that elicit an RNA interference silencing of the overexpressed genes. Using high throughput methodologies, we have constructed a library of sequence-verified hairpins that target most human genes (5). Constructs from the library have been used for the systematic validation of breast cancer molecular profiling data in ErbB-2 positive BT 474 cells. The overall objective of this proposal is to identify hairpins that have inhibitory effects on breast cancer progression in *in vivo* models as this would add to our understanding of the genes involved in breast cancer and identify their products as potential small molecule therapeutic targets. It would also pave the way for testing of the hairpins that we identify as gene therapeutics.

The underlying hypothesis of this proposal is that functional genomic screening with DNA-encoded RNA interference can identify those genes that breast cancer cells actually rely on to cause cancer. We will systematically target the most commonly overexpressed genes in breast cancer cells especially those that are overexpressed in association with ErbB-2 amplification. Many of these genes are likely to be involved in granting some sort of survival advantage to these cells during cancer progression. Silencing key genes in this group should reverse the cancer phenotype. In this way, we expect to gain insights into which genes are actually required by breast cancer cells to cause cancer and would therefore make excellent therapeutic targets. Moreover, it may contribute directly to the development of gene-based therapeutics by identifying short hairpin RNAs that could someday be used clinically.

Body

An RNAi screen targeting genes overexpressed in ERBB2-positive breast cancer

Since ERBB2-positive breast cancer patients with metastatic disease show overall Herceptin response rates of between ~10 and 25%, it is likely that factors other than ERBB2 itself must affect the aggressiveness and poor therapy response in these tumors. So that genes required for the survival of ERBB2 breast cancer cells could be identified, we performed a functional RNAi screen of 144 genes previously identified by expression profiling meta-analyses to be upregulated in ERBB2-positive breast cancer [1-6]. A total of 323 short-hairpin RNAs (shRNAs) expr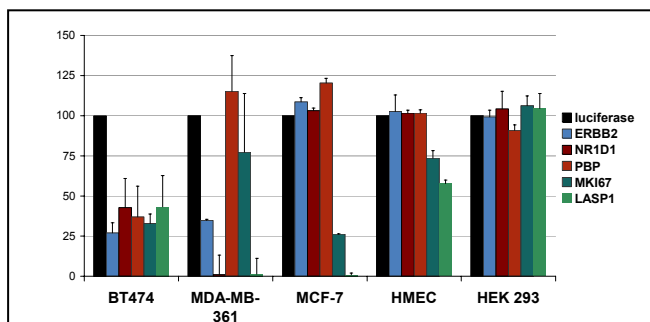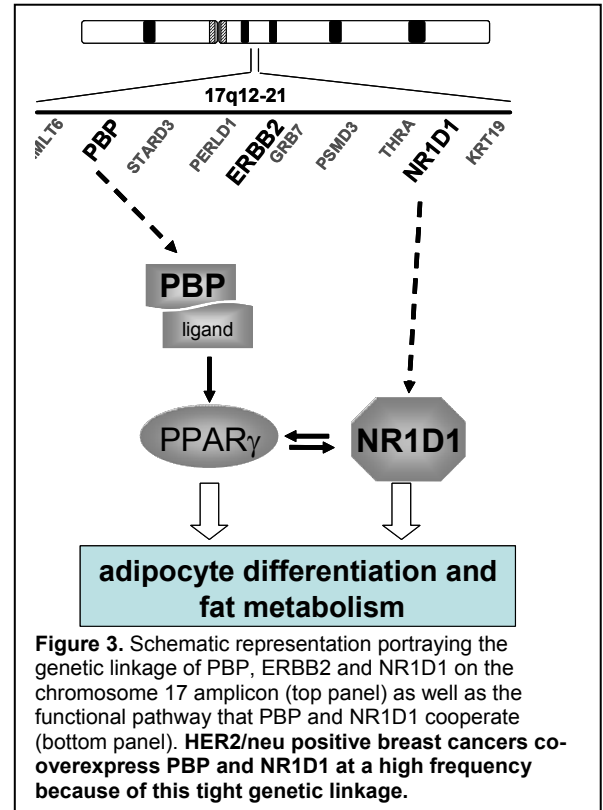essed from the pSHAG-MAGIC 2 (pSM2) vector were used to down regulate or "knock down" the expression of these genes in the well-characterized, ERBB2-positive BT474 breast cancer cell line. To monitor the effects of shRNAs on the cells, we used alamarBlue, a colorimetric indicator of cell viability (**Fig. 2A**). Eleven shRNAs resulted in more than a 50% decrease in cell viability (**Fig. 2B**). This group contained an ERBB2 shRNA that caused the largest decrease in alamarBlue staining (~70%). This was expected since the role of ERBB2 in breast cancer cell proliferation is well established [7]. Other genes which when knocked down produced more than a 50% decrease in cell viability included genes with known roles in cancer cell proliferation. These included CA9[8], BNIP3L [9], KI67, and TPD52 (**Fig. 2B**). Knockdown of ERBB3, another member of the EGFR family that heterodimerizes with ERBB2 as its preferred partner [7], also had a modest impact on cell viability, resulting in ~31% of alamarBlue decrease (data not shown). That the screen identified these genes known to be important to breast cancer cell proliferation validates the utility of this approach in identifying genes important for the survival of this type of cancer.

Symbol	Name	BT474	HMEC
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	++++	-
PBP	PPAR binding protein PPARBP	++++	-
NR1D1	Nuclear receptor subfamily 1, group D, member 1	++++	-
MAP2K6	Mitogen-activated protein kinase kinase 6	++++	-
SPINT1	Serine protease inhibitor, Kunitz type 1	++++	-
PFN2	Profilin 2	++++	+
MKI67	Antigen identified by monoclonal antibody Ki-67	++++	++
TPD52	Tumor protein D52	++++	++
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	++++	++
H2AFY	H2A histone family, member Y	++++	++
LASP1	LIM and SH3 protein 1	++++	+++
SIAT4C	Sialyltransferase 4C	+++	-
SFRS7	Splicing factor, arginine/serine-rich 7, 35kDa	+++	-
RPL19	Ribosomal protein L19	+++	+
CA9	Carbonic anhydrase IX	+++	++
FADS2	Fatty acid desaturase 2	++	ND
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3	++	ND
GRB7	Growth factor receptor bound protein 7	++	ND
STARD3	StAR-related lipid transfer (START) domain containing 3	++	ND

Figure 2. An RNAi screen targeting genes overexpressed in breast cancer. (**A**) BT474 breast cancer cells were transfected with 323 shRNA constructs targeting 144 overexpressed genes. Non-specific shRNA constructs targeting the firefly luciferase gene were used as negative controls and shRNAs targeting ERBB2 were used as positive controls. All shRNAs were co-transfected with a construct expressing green fluorescent protein (GFP). After four days cell proliferation was measured using alamarBlue (Biosource). AlamarBlue results were normalized to transfection efficiency determined from GFP expression and are represented as percentage of normalized luciferase control. Three transfection mixes were produced for each shRNA. Each reaction was transfected into triplicate wells. The averages and standard deviations of (9 parallel) cultures treated with each shRNA were calculated. Results shown are sorted on the basis of the effect. **B**. **Summary of best hits.** Crosses indicate magnitude of effect on cells after shRNA-mediated knockdown compared to the luciferase shRNA control; ND: not determined on HMEC; Genes above the bold line produced more than 50% decrease in alamarBlue staining on BT474 cells. shRNAs shaded in blue target genes that have known roles in adipogenesis. These hits have been confirmed in a second round of transfections.

Surprisingly, three of the shRNAs that had the most dramatic effect , targeted genes that had been shown previously to play roles in adipogenesis. These included PPARγ binding protein (PBP)[10], nuclear receptor subfamily 1, group D, member 1 (NR1D1 or RevErbα)[11], and the MAP kinase, MAP2K6[12]. Two others, fatty acid desaturase 2 (FADS2)[13] and STARD3[14], which had less of an effect, also are involved in this process. PBP and NR1D1 are particularly interesting. They are genetically linked, both residing on the 17q12-21 amplicon, together with ERBB2 (**Fig. 3**). They are also functionally linked; a number of studies have shown them to be required for transcriptional regulation of lipid metabolism and adipocyte differentiation. PBP is a co-activator of PPARγ. PPARγ has been extensively studied and positively regulates adipogenic genes including NR1D1. NR1D1, which is an orphan nuclear receptor, is relatively poorly understood but is thought to normally act as a negative regulator of transcription. It may act by inhibiting expression of anti-adipogenic genes[15] or upregulating genes such as PPARγ ([11]and **Fig. 3**).

Although we would have liked to carry out studies of the effect of each shRNA on a more extensive panel of breast cancer cell types, we were able to gain some idea of the specificity of effect for the ERBB2, PBP and NR1D1 shRNAs. The best 15 shRNA hits from the initial screen were transfected into other

Figure 3. Schematic representation portraying the genetic linkage of PBP, ERBB2 and NR1D1 on the chromosome 17 amplicon (top panel) as well as the functional pathway that PBP and NR1D1 cooperate (bottom panel). **HER2/neu positive breast cancers co-overexpress PBP and NR1D1 at a high frequency because of this tight genetic linkage.**

breast cancer cell lines including the MDA-MB-361 (ERBB2-positive), MCF-7 (ERBB2-negative), normal human mammary epithelial cells (HMECs) a non-breast derived tumorigenic cell line, HEK 293FT cells and BT474s(ERBB2-positive, ER) . Cells were assayed using the same approach as in the original screen. The ERBB2 shRNA specifically had an impact on the two ERBB2-positive breast cancer cell lines, BT474 and MDA-MB-361 (**Fig. 4**), as expected. Interestingly, the NR1D1 shRNA resulted in decreased viability of the same two cell lines yet was without effect on MCF7s and HMECs (**Fig. 4**). PBP inhibited BT474 proliferation but had no effect on MDA-MB-361 cells. This may be due to higher levels of PBP dosage in these cells which also possess the 17q12-21 amplicon. We have confirmed the gene silencing activity of the PBP and NR1D1 shRNAs both at the message and the protein levels. Both shRNAs decrease levels of mRNA approximately 60% and since the abundance of mRNAs from closely linked genes does not change we are confident that these shRNAs act at the post-transcriptional level. siRNAs to NR1D1 have also been tested yet have less of an effect (data not shown).

Figure 4. Screen hits tested for effects on other cell lines. An ERBB2-positive cell line (MDA-MB-361), a non-ERBB2 overexpressing cell line (MCF-7), normal mammary epithelial cells (HMEC) and a non-breast cell line (HEK 293) were transfected with shRNAs. Experiments were performed as in Fig.1. Error bars represent standard deviations from triplicate experiments.

NR1D1 links the circadian clock and metabolism
Although originally linked to regulation of adipocyte differentiation and lipid metabolism [16, 17], NR1D1 has been recently identified as a major component of the circadian clock [18, 19] which coordinates animal behavior and physiology with an approximately 24 hr cycle. Deregulation of circadian rhythms has been associated with jet lag, sleep disorders, metabolic disruptions and tumorigenesis. This system is centrally controlled by the suprachiasmatic nucleus (SCN),
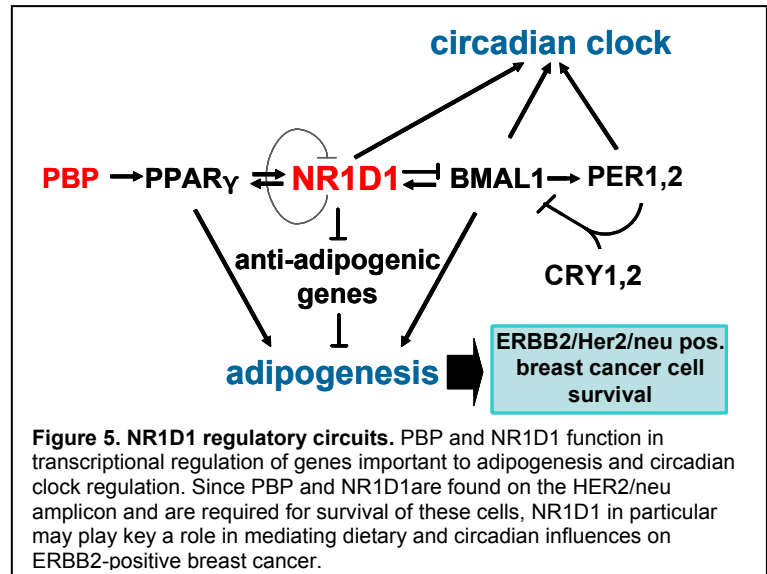
which influences the activity of peripheral clocks through the nervous system. At the molecular level, the circadian clock is maintained by a complex system of feedback loops involving a number of transcription factors. As shown in **Figure 5**, NR1D1 has effects on circadian regulation through its suppression of BMAL1 (Brain and Muscle Arnt-like protein 1) via recruitment of the NCoR/HDAC3 deacetylase [20]. BMAL1 heterodimerizes with another circadian component, CLOCK (Circadian Locomoter Output Cycles Kaput protein). BMAL1-CLOCK heterodimers can induce expression of NR1D1 [19] as well as of another set of clock genes, the PER1 (period homolog 1) and 2 and CRY1 (cryptochrome 1) and 2 genes, thereby forming the positive limb of the circadian clock.

Figure 5. NR1D1 regulatory circuits. PBP and NR1D1 function in transcriptional regulation of genes important to adipogenesis and circadian clock regulation. Since PBP and NR1D1are found on the HER2/neu amplicon and are required for survival of these cells, NR1D1 in particular may play key a role in mediating dietary and circadian influences on ERBB2-positive breast cancer.

BMAL1 was also found to promote adipocyte differentiation [21], further supporting the notion of a direct relationship between circadian clock and metabolism. The PER and CRY proteins heterodimerize to inhibit BMAL1-CLOCK mediated transcription, thus acting as a negative circadian component [22]. DNA binding of BMAL1-CLOCK is strongly enhanced by low NAD/NADH ratio, implying for one more link between circadian rhythm and metabolism [23]. **Because of its regulation of fat storage, NR1D1 has been proposed as a potential link between the circadian clock and cellular metabolism [24].**

It is of note that a number of studies have shown that disruption of the circadian cycle accelerates malignant growth [25-27], though the molecular mechanisms remain essentially unknown. Although there has been no direct evidence showing how NR1D1 overexpression could influence breast cancer aggressiveness, this gene has been proposed as a potential druggable target for breast cancer therapy [28]. Interestingly, the PER family of genes was found to be methylated in a set of breast tumors where ERBB2 was overexpressed [29]. NR1D1 overexpression and PER methylation in ERBB2-positive tumors may be a consequence of their functional relationship, since NR1D1 suppresses BMAL1 and therefore PER1 expression.

PBP and NR1D1 knockdown results in increased apoptosis in BT474 cells
Since reduced proliferation can result from slowed growth, quiescence or increased cell death, the cause of the decreased proliferation of ERBB2-positive cells due to PBP and NR1D1 knockdown was determined. Cells were transfected with PBP, NR1D1, ERBB2 and Luciferase shRNAs. GFP was used as a marker of transfection. Cells were monitored for three days and imaged using a InCell high-content imaging system (General Electric), in order to quantify the number of transfected cells based on GFP expression (data not shown). Results showed a time dependent decrease in the number of cells transfected with the NR1D1 shRNA. A similar though smaller effect was observed with the PBP-transfected cells. That transfected cells were lost from the population implied that cell death was responsible for the decrease in alamarBlue signal seen in the primary screen. ERBB2 knockdown did not have the same phenotypic effect, since the number of transfected cells did not decrease with time (data not shown) which agrees with some other studies showing that the major effect of ERBB2 knockdown is cytostatic [30-32]. These results, which indicate a difference in the genetic phenotype, imply that PBP and NR1D1 may not function in the same biological pathway as ERBB2, although they all map closely together.
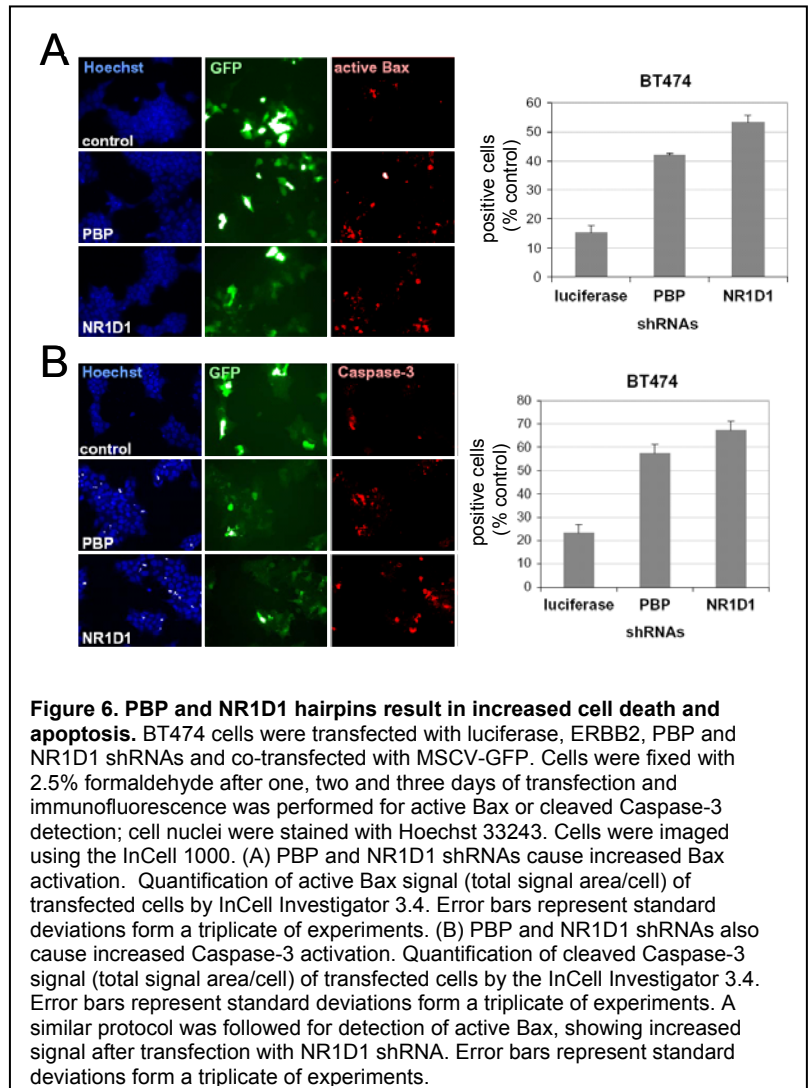
In order to examine whether cell death caused by the PBP and NR1D1 shRNAs is due to apoptosis, we employed detection of activated Bax and cleaved Caspase-3 by immunofluoresence. When active, Bax translocates to mitochondria inducing changes in mitochodrial membrane permeability that enables cytochrome C to be released in the cytoplasm and triggers the Caspase cascade. Caspase-3 is a major component of the apoptotic machinery and is proteolytically cleaved to its active form by a variety of stimuli promoting cell apoptosis. Images of shRNA-transfected cells and digital quantification of the active Bax signal (**Fig. 6A**) showed that cells transfected with the NR1D1 and PBP shRNAs, but not with the ERBB2 shRNA, exhibited increased apoptosis compared to the control. Images of shRNA-transfected cells and digital quantification of the Caspase-3 signal (**Fig. 6B**) showed that cells transfected with the NR1D1 and PBP shRNAs also exhibited increased apoptosis compared to the control. PBP knockdown increased Caspase-3 cleavage 3-fold. This effect was smaller than 5-fold increase caused by the NR1D1 shRNA and is consistent with the observed differences in live cell counts (data not shown). This result could be attributed to either the higher efficiency of the NR1D1 shRNA or to a more central role of NR1D1 in BT474 survival. These results show that NR1D1 and PBP knockdown cause apoptotic cell death in BT474 cells. That NR1D1 shRNA had a more profound effect on cell viability and increased apoptosis, compared to PBP and ERBB2, points to the importance of NR1D1 to BT474 cell viability.
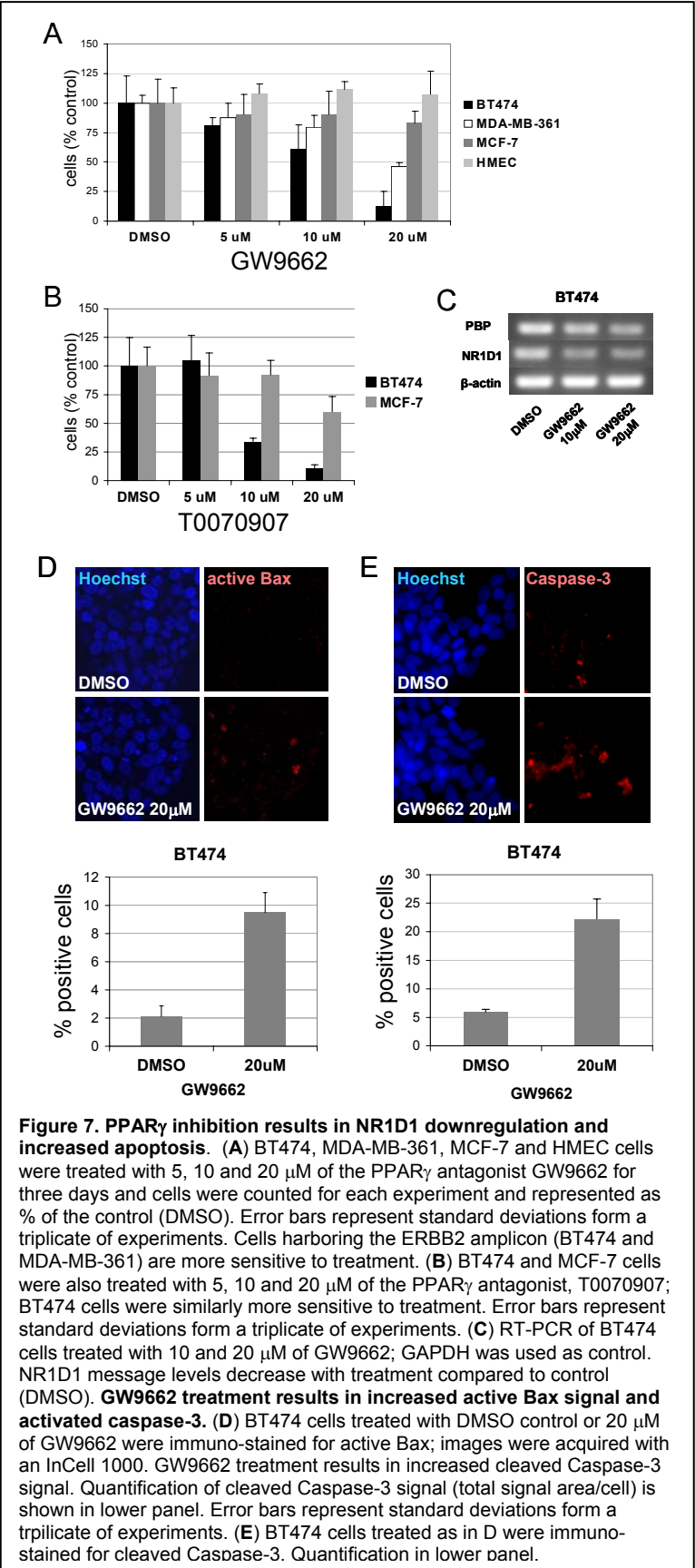
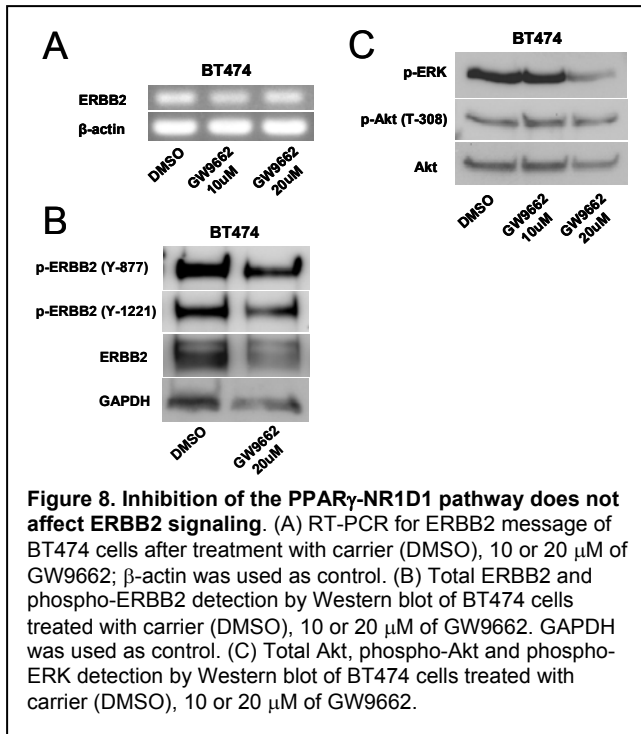**PPARγ antagonists result in NR1D1 knockdown and apoptosis induction of amplicon-possessing cells**

NR1D1 and PBP are both functionally related to PPARγ. PBP is a co-activator of PPARγ. NR1D1 is a target of PPARγ and has been shown to positively regulate PPARγ expression. PPARγ transcriptional activity is activated by a number of different fatty acid metabolites. Several agonists and antagonists are available. To determine whether PPARγ is a mediator of the PBP-NR1D1 pathway in the amplicon-positive breast cancer cells, we treated BT474, MDA-MB-361, MCF-7 and HMEC cells with several concentrations of two antagonists that bind irreversibly to PPARγ, GW9662 and T0070907 (**Fig. 7A,B**). Although both drugs were toxic to all cells, BT474 and MDA-MB-361 cells were more sensitive to treatment than MCF-7 and HMEC cells (**Fig. 7A,B**). That these cell lines are more sensitive is similar to the effects seen with PBP and NR1D1 shRNAs. GW9662 appeared to affect PPARγ signaling since treatment resulted in decreased NR1D1 expression, as expected (**Fig. 7C**) and increased apoptosis of BT474 cells, as shown by active Bax (**Fig. 7D**) and cleaved Caspase-3 (**Fig. 7E**). Hence, PPARγ inhibitors have the same phenotypic effects and the same pattern of specificity as the

Figure 6. PBP and NR1D1 hairpins result in increased cell death and apoptosis. BT474 cells were transfected with luciferase, ERBB2, PBP and NR1D1 shRNAs and co-transfected with MSCV-GFP. Cells were fixed with 2.5% formaldehyde after one, two and three days of transfection and immunofluorescence was performed for active Bax or cleaved Caspase-3 detection; cell nuclei were stained with Hoechst 33243. Cells were imaged using the InCell 1000. (A) PBP and NR1D1 shRNAs cause increased Bax activation. Quantification of active Bax signal (total signal area/cell) of transfected cells by InCell Investigator 3.4. Error bars represent standard deviations form a triplicate of experiments. (B) PBP and NR1D1 shRNAs also cause increased Caspase-3 activation. Quantification of cleaved Caspase-3 signal (total signal area/cell) of transfected cells by the InCell Investigator 3.4. Error bars represent standard deviations form a triplicate of experiments. A similar protocol was followed for detection of active Bax, showing increased signal after transfection with NR1D1 shRNA. Error bars represent standard deviations form a triplicate of experiments.

PBP and NR1D1 shRNAs, which may occur, at least in part, through the downregulation of NR1D1 and indicate that these three genes function as part of a single pathway that is required for breast cancer cell survival. **We refer to this as the NR1D1 pathway**. That we found that drugs targeting PPARγ had an effect on cancer cells is not novel. An enormous literature exists on the use of PPARγ agonists in treating cancer (for review [33]). Fewer studies have been conducted with antagonists. In both cases, there are conflicting results and no unified picture has emerged. Our results are unique since they point to effects of PPARγ antagonists on cells with a specific genotype. Irrespective of potential therapeutic use these drugs are useful as tools, since they affect the same pathway and do not suffer from issues related to transfection.

Figure 7. PPARγ inhibition results in NR1D1 downregulation and increased apoptosis. (**A**) BT474, MDA-MB-361, MCF-7 and HMEC cells were treated with 5, 10 and 20 μM of the PPARγ antagonist GW9662 for three days and cells were counted for each experiment and represented as % of the control (DMSO). Error bars represent standard deviations form a triplicate of experiments. Cells harboring the ERBB2 amplicon (BT474 and MDA-MB-361) are more sensitive to treatment. (**B**) BT474 and MCF-7 cells were also treated with 5, 10 and 20 μM of the PPARγ antagonist, T0070907; BT474 cells were similarly more sensitive to treatment. Error bars represent standard deviations form a triplicate of experiments. (**C**) RT-PCR of BT474 cells treated with 10 and 20 μM of GW9662; GAPDH was used as control. NR1D1 message levels decrease with treatment compared to control (DMSO). **GW9662 treatment results in increased active Bax signal and activated caspase-3.** (**D**) BT474 cells treated with DMSO control or 20 μM of GW9662 were immuno-stained for active Bax; images were acquired with an InCell 1000. GW9662 treatment results in increased cleaved Caspase-3 signal. Quantification of cleaved Caspase-3 signal (total signal area/cell) is shown in lower panel. Error bars represent standard deviations form a trpilicate of experiments. (**E**) BT474 cells treated as in D were immuno-stained for cleaved Caspase-3. Quantification in lower panel.

9

Figure 8. Inhibition of the PPARγ-NR1D1 pathway does not affect ERBB2 signaling. (A) RT-PCR for ERBB2 message of BT474 cells after treatment with carrier (DMSO), 10 or 20 µM of GW9662; β-actin was used as control. (B) Total ERBB2 and phospho-ERBB2 detection by Western blot of BT474 cells treated with carrier (DMSO), 10 or 20 µM of GW9662. GAPDH was used as control. (C) Total Akt, phospho-Akt and phospho-ERK detection by Western blot of BT474 cells treated with carrier (DMSO), 10 or 20 µM of GW9662.
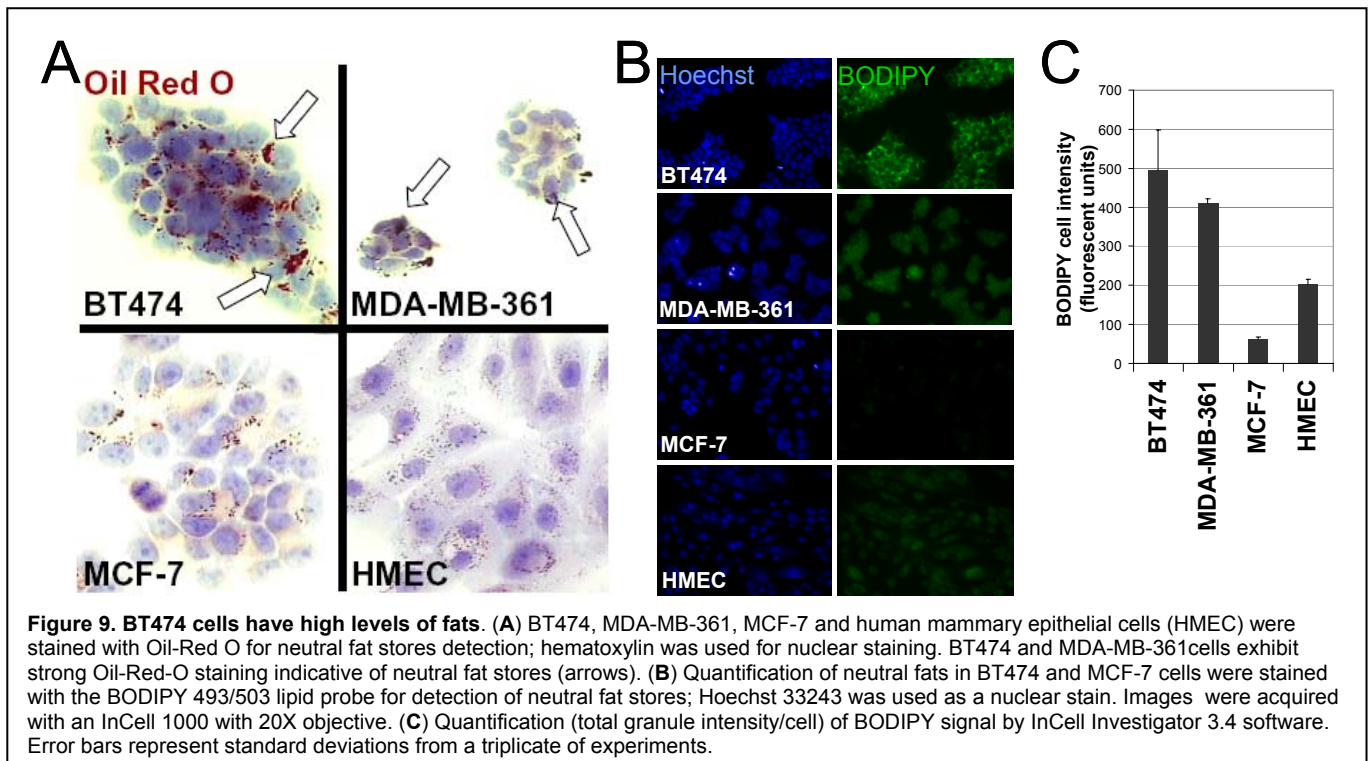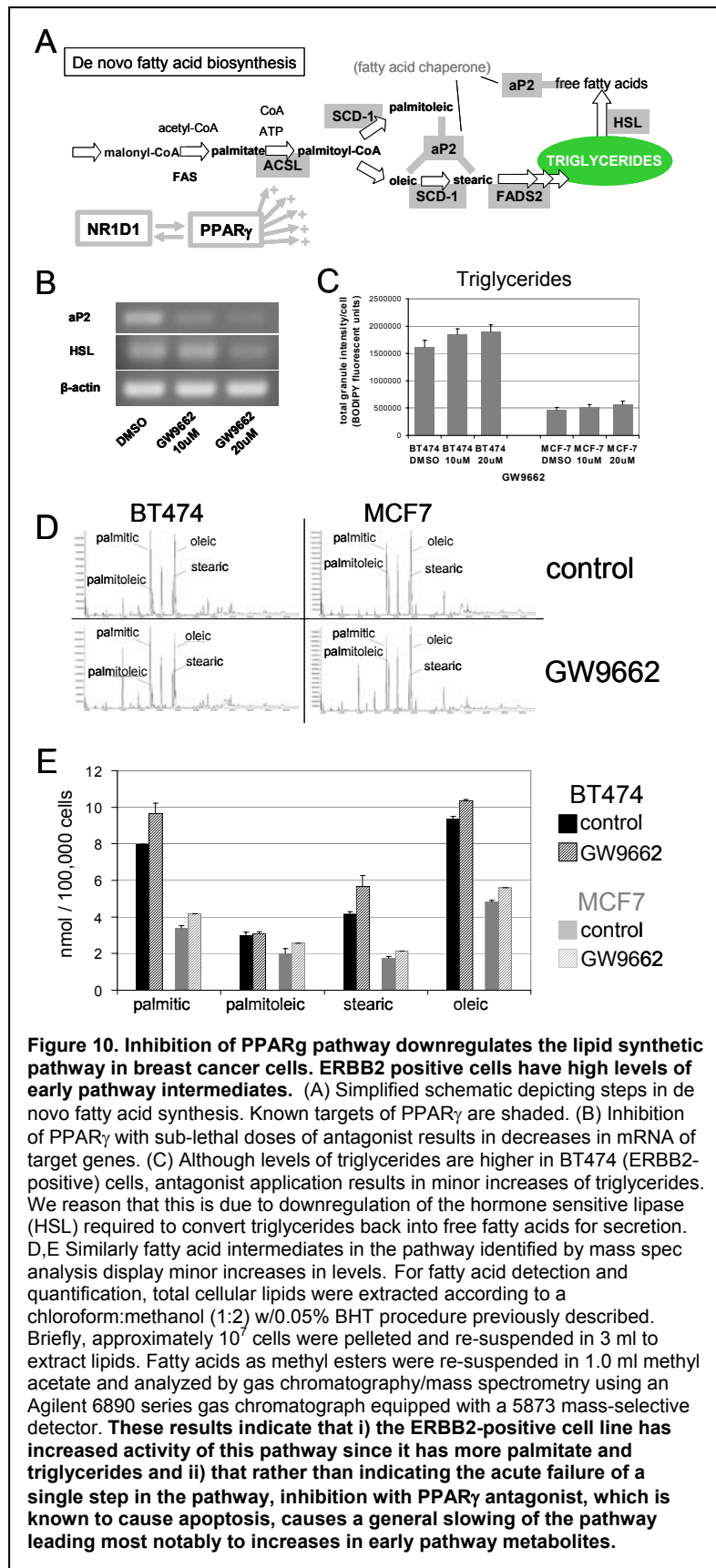
Inhibition of PBP-PPARγ-NR1D1 does not affect ERBB2 signaling

Since shRNAs and PPARγ antagonists specifically induced apoptosis in ERBB2-positive breast cancer cells, it was necessary to investigate whether ERBB2 signaling is affected by inhibiting the PBP- PPARγ-NR1D1 pathway. To do this, we examined ERBB2 signaling in BT474 cells after treatment with the PPARγ antagonist GW9662. Neither ERBB2 expression (**Fig. 8A**), nor ERBB2 phosphorylation on two tyrosine residues that are targets of phosphorylation (**Fig. 8B**) were significantly affected by the GW9662 treatment. It is widely accepted that the two major effectors activated by ERBB2 are the PI3K-Akt and the MAPK pathways. Akt phosphorylation is not altered after treatment with the PPARγ antagonist (**Fig. 8C**), in agreement with the ERBB2 phosphorylation status. However, a high dose of GW9662 resulted in decreased ERK phosphorylation (**Fig. 8C**), contrary to ERBB2 phosphorylation status. The latter may be a direct effect of PPARγ inhibition, since it has been shown that PPARγ positively regulates ERK signaling ([34, 35]). Although they are somewhat preliminary, these results indicate that a simple relationship does not exist between the PPARγ-NR1D1 pathway and ERBB2. Specific Aim 1 is directed at further analyzing potential interactions between ERBB2 and the NR1D1 pathway.

Figure 9. BT474 cells have high levels of fats. (**A**) BT474, MDA-MB-361, MCF-7 and human mammary epithelial cells (HMEC) were stained with Oil-Red O for neutral fat stores detection; hematoxylin was used for nuclear staining. BT474 and MDA-MB-361cells exhibit strong Oil-Red-O staining indicative of neutral fat stores (arrows). (**B**) Quantification of neutral fats in BT474 and MCF-7 cells were stained with the BODIPY 493/503 lipid probe for detection of neutral fat stores; Hoechst 33243 was used as a nuclear stain. Images were acquired with an InCell 1000 with 20X objective. (**C**) Quantification (total granule intensity/cell) of BODIPY signal by InCell Investigator 3.4 software. Error bars represent standard deviations from a triplicate of experiments.

Figure 10. Inhibition of PPARg pathway downregulates the lipid synthetic pathway in breast cancer cells. ERBB2 positive cells have high levels of early pathway intermediates. (A) Simplified schematic depicting steps in de novo fatty acid synthesis. Known targets of PPARγ are shaded. (B) Inhibition of PPARγ with sub-lethal doses of antagonist results in decreases in mRNA of target genes. (C) Although levels of triglycerides are higher in BT474 (ERBB2-positive) cells, antagonist application results in minor increases of triglycerides. We reason that this is due to downregulation of the hormone sensitive lipase (HSL) required to convert triglycerides back into free fatty acids for secretion. D,E Similarly fatty acid intermediates in the pathway identified by mass spec analysis display minor increases in levels. For fatty acid detection and quantification, total cellular lipids were extracted according to a chloroform:methanol (1:2) w/0.05% BHT procedure previously described. Briefly, approximately 10^7 cells were pelleted and re-suspended in 3 ml to extract lipids. Fatty acids as methyl esters were re-suspended in 1.0 ml methyl acetate and analyzed by gas chromatography/mass spectrometry using an Agilent 6890 series gas chromatograph equipped with a 5873 mass-selective detector. **These results indicate that i) the ERBB2-positive cell line has increased activity of this pathway since it has more palmitate and triglycerides and ii) that rather than indicating the acute failure of a single step in the pathway, inhibition with PPARγ antagonist, which is known to cause apoptosis, causes a general slowing of the pathway leading most notably to increases in early pathway metabolites.**

The PBP-PPARγ-NR1D1 pathway alters fat metabolism in breast cancer cells

PPARγ and NR1D1 are major regulators of adipogenesis and lipid homeostasis. Up-regulation of these genes promotes lipid accumulation in fat stores during adipocyte differentiation [11, 15, 16, 36]. Since both genes are likely to have a number of targets, it was important to determine whether this function was relevant to the breast cancer cells. As shown in **Figures 9** and **10**, several lines of evidence indicate that ERBB2-positive breast cancer cells have increased levels of fat. BT474 and MDA-MB-361cells exhibit strong staining with Oil-Red-O, which stains neutral fat stores, when compared to MCF-7 and HMEC cells (**Fig. 9A**). BODIPY 493/503, a fluorescent probe of cellular lipids gave similar results. (**Fig. 9B**) Quantification of fluorescent signal from BODIPY-stained neutral fats (total granule intensity/cell) was performed by InCell 1000 image capture and quantification with InCell Investigator 3.4 software. ERBB2-positive BT474 and MDA-MB-361cells showed a 2-fold increase in fat accumulation in lipid stores, when compared to HMEC cells and a 5-fold increase when compared to MCF-7(**Fig. 9B**). Thin layer chromatography and densitometry confirmed that the neutral fat stores were triglycerides (data not shown).

When quantified by mass spectrometry, differences were also observed in total cellular fats. Since we were primarily interested in learning why the EBRB2-positive cell line was sensitive to treatment with shRNAs and PPARγ antagonists while the EBRB2-negative cell line was relatively resistant, we focused on differences between these two lines. Total cellular lipids from both were extracted and analyzed by gas chromatography/mass spectrometry[37]. BT474 cells were found to contain slightly more than twice the amount of total fats when compared to MCF-7 cells (28 nmol/10^5 cells versus 13 nmol/10^5 cells, data not shown). It is not surprising that BT474s have high total fat content because the gene encoding fatty acid synthase is

overexpressed in these cells [38-41]. That these cells store the increased fats as triglycerides is somewhat unexpected and may provide a rationale for the requirement of PBP and NR1D1 overexpression in this cell type (see below).

To learn clues about the trigger of apoptosis after inhibition of the PBP-PPARγ-NR1D1 pathway, we examined cells for changes in the fat metabolic pathway. As shown in **Figure 10B**, inhibition of the PBP-PPARγ-NR1D1 pathway affected downstream targets involved in lipid metabolism in the breast cancer cells. Expression levels of aP2 (FABP4, fatty acid binding protein 4), one of the most commonly used markers of adipogenesis and of HSL (hormone sensitive lipase) both of which are positively regulated by PPARγ during adipocyte differentiation, were downregulated upon use of the PPARγ antagonist GW9662 (**Fig. 10B**). Although the glucose transporter, GLUT4, was also downregulated, we have shown that diminished glucose uptake is not an explanation for apoptosis onset (data not shown). We then examined the effect that inhibition of the pathway would have on fat accumulation. Treatment with a sublethal concentration of PPARγ antagonist or administration of PBP and NR1D1 shRNA treatment increased fat stores (**Fig. 10C** and data not shown). Levels of several intermediates between palmitate and triglyceride stores also showed modest increases in both BT474 and MCF-7 cells (**Fig. 10D-E**). Although initially surprising, this result is likely due to the downregulation of HSL, a lipase that is responsible for breakdown of triglycerides. **We hypothesize that the major effect of inhibition of the PBP-PPARγ-NR1D1 pathway is a generalized inhibition of the fat storage processes that are usually coordinated by these transcription factors. That the effect is more profound in ERBB2-positive breast cancer cells reflects the overall higher activity of the fatty acid synthesis pathway in these cells.**

Apoptosis due to PBP-PPARγ-NR1D1 inhibition is consistent with endogenous palmitate toxicity. The relatively high levels of activity of the fatty acid synthesis pathway in the ERBB2-positive breast cancer cells suggested a possible trigger of apoptosis in these cells. The end-product of FASN activity, palmitate, and other saturated fatty acids like it are toxic to cells, generating a variety of apoptotic signals, including ceramide synthesis[42] and reactive oxygen species[43] (for review see [44]). Studies in a variety of cell types, including breast cancer cell lines [45, 46] suggest that this lipotoxicity is specific for saturated fatty acids and that exogenous or endogenously generated unsaturated
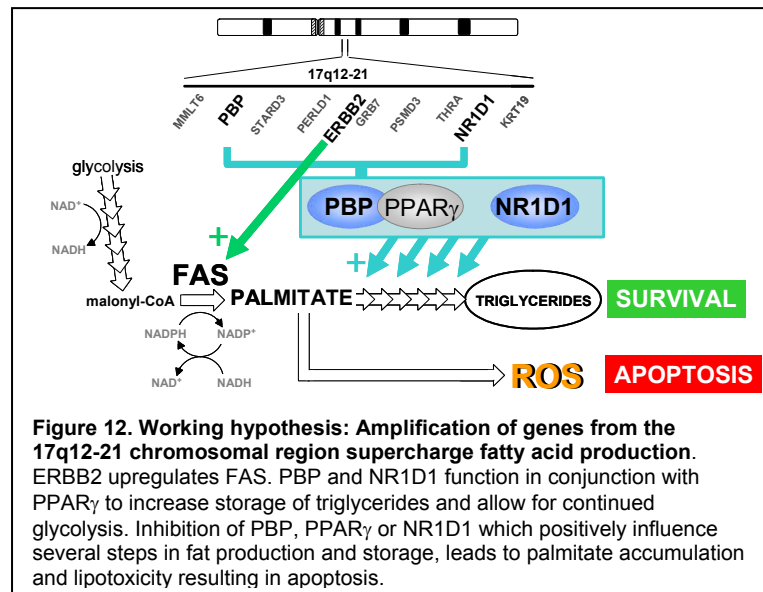

Figure 11. ERBB2 positive BT474 cells are sensitivr to palmiatate. Inhibition of the PBP-PPARγ-NR1D1 pathway oxidizes cells. (A,B) BT474 cells were transfected with luciferase, PBP and NR1D1 hairpins or treated with GW9662 (C,D) and stained with Hydroethidine for detection of O_2- species; images were acquired by the InCell 1000 (top panel) and signal intensity/cell was quantified using the InCell Analyzer 3.4 software (bottom panel). Error bars represent standard deviations from a triplicate of experiments. Both PBP and NR1D1 hairpins increased Hydroethidine staining. (E,F) BT474 cells are sensitive to exogenously added palmitate. The effect on cells is exacerbated by GW9662. Cultures were incubated for 3 days with concentrations shown.

FASN rescue palmitate-induced apoptosis by promoting palmitate incorporation into triglyceride. In tests, BT474 cells were found to be more sensitive than MCF-7 cells to exogenously added palmitate (**Fig. 11E-F**). The effect on cells was exacerbated by GW9662 (**Fig. 11E-F**) but was largely unaffected by a ceramide synthesis inhibitor (data not shown). These results support the idea that BT474 cells have near toxic levels of endogenously produced palmitate which lead to apoptosis when the triglyceride storage pathway is downregulated.

In the studies in Chinese hamster ovary cells, intracellular palmitate accumulation induces apoptosis through the generation of reactive oxygen species (ROS)[43]. To test whether PBP-PPARγ-NR1D1 knockdown resulted in ROS production, we measured ROS by using hydroethidine after BT474 treatment with the PBP and NR1D1 shRNAs (**Fig. 11A-B**) or with the PPARγ antagonist GW9662 (**Fig. 11C-D**). Results in both cases showed that cells showed increased production of ROS species since both shRNA and antagonist treatments lead to increased fluorescence of hydroethidine. Taken together these results indicate that apoptosis due to PBP-PPARγ-NR1D1 inhibition is consistent with endogenous palmitate toxicity. Based on our results to date, we hypothesize that increased activity of the PBP-PPARγ-NR1D1pathway enables ERBB2 positive breast cancer cells to convert fatty acids to triglycerides, allowing these cells to avert lipotoxicity such that they can continue to catabolize glucose. The tight genetic linkage between NR1D1 and ERBB2, suggests that **ERBB2 positive breast cancer cells are genetically programmed to depend on fatty acid synthesis and triglyceride storage for energy production.**

A working model consistent with our results is shown below (Fig12).

Figure 12. Working hypothesis: Amplification of genes from the 17q12-21 chromosomal region supercharge fatty acid production. ERBB2 upregulates FAS. PBP and NR1D1 function in conjunction with PPARγ to increase storage of triglycerides and allow for continued glycolysis. Inhibition of PBP, PPARγ or NR1D1 which positively influence several steps in fat production and storage, leads to palmitate accumulation and lipotoxicity resulting in apoptosis.

Key Research Accomplishments

Year 1.

- Adopted a new vector and shRNA production methodology

- Created a set of encoded hairpins targeted against genes overexpressed in ErbB-2 positive breast tumors

- Developed a relatively rapid and reliable method for quantification of proliferation changes due to shRNA expression in cells

- Identified approximately 30 genes that negatively impact the growth of Erb-B2 positive breast cancer cells

Year 2.

- Tested shRNAs that negatively impact the growth of Erb-B2 positive breast cancer cells for effects on normal cells

- Tested shRNAs that negatively impact the growth of Erb-B2 positive breast cancer cells for effects on other breast cancer cell lines

- Identified approximately 20 shRNAs from the original positives from year 1 that specifically inhibit Erb-B2 positive breast cancer cells target genes. These genes are potential chemotherapeutic targets.

- The PBP and NR1D1 genes are related to adipogenesis. Using small molecule inhibitors and reporter experiments, we have developed a working hypothesis that adipogenic genes may be required for breast cancer proliferation. This is likely to be due to its effect on countering cellular hypoxia.

Year 3.

- **We have examined the relationship of the NR1D1-PBP-PPARγ pathway to ERBB2 signaling.**

- **We have identified a likely mechanism for triggering apoptosis in the her-2/neu positive breast cancer cells.**

- **We have sought funding for the continuation of this work.**

- **We have prepared a manuscript that will be submitted presently on his work.**

Year 3 Reportable Outcomes

Manuscripts:

Evans, S. S. Markham, A. Torres, A. Kourtidis, and D. Conklin. 2006. An improved minimum description length learning algorithm for nucleotide sequence analysis. *Signals, Systems and Computers*, Oct.-Nov. 2006. 1843-1850

Kourtidis, A., Eiffert, C., and Conklin, D.S., 2006 RNAi Applications in Target Validation. *Ernst Schering Res Found Workshop Systems Biology.* 2007;(61):1-21

Evans, S., A. Kourtidis, T. S. Markham, J. Miller, D. Conklin and A. Torres., 2007, microRNA target detection and analysis for genes related to breast cancer using MDLcompress. *EURASIP Journal on Bioinformatics and Systems Biology.* Volume 2007 (2007), Article ID 43670

Begley, U., M. Dyavaiah, A. Patil, J. P. Rooney, D. DiRenzo, C.M. Young, D.S. Conklin, R.S. Zitomer and T.J. Begley 2007. Trm9 Catalyzed tRNA Modifications Link Translation to the DNA Damage Response. *Mol. Cell (in press)*

Lastro, M.L., Kourtidis, A. Farley, K. and Conklin, D.S. 2007. xCT expression reduces the early cell cycle requirement for calcium. *Cell. Signal. (in press)*

Ranganathan, A.C., A. Kourtidis, S. Ojha, A. Kourtidis, D. S. Conklin and J. A. Aguirre-Ghiso. , 2007, Inducible activation of PERK reveals an intensity-dependent tumor growth inhibitory function. (*submitted*)

Adam, A.P., A. George, B.V. Iglesias, A. Kourtidis, D. S. Conklin and J. A. Aguirre-Ghiso. (2007) Regulation by p38^{SAPK} of a transcription factor network required for tumor cell quiescence. (*in preparation*)

A. Kourtidis, R.D. Carkner, M.J. Brosnan and D.S. Conklin, 2007, An RNAi screen identifies adipogenesis as a survival pathway for breast cancer cells with the *HER2/neu* amplicon. (*in preparation*)

Presentations:

Petunias hold the keys to new cancer research and therapies (invited presentation) American Cancer Society "Wellness Day" May 2007

Functional genomics in mammalian cells (invited presentation), University of Pittsburgh Cancer Center, February 2007

Development of cell lines, tissue or serum repositories:

As was proposed, the collection of shRNA constructs targeting overexpressed genes that contribute to breast cancer tumorigenicity will undoubtedly be useful to many investigators and will be made available as soon as testing is complete.

Funding applied for based on work supported by this award:

Submission of an RO1 is planned for February 2008 with preliminary results produced from this project.

Additionally, the following proposals made possible by this award have been submitted:

American Cancer Society (Conklin) $850,000 total
 "NR1D1 Pathway in Breast Cancer"

The major goal of this project is to determine the role that this pathway plays in breast cancer.
Role: **Principal Investigator**

Employment or research opportunities applied for and/or received based on experience/training supported by this award:

None in this year.

Year 3 Conclusions

Year 1 of funding resulted in the collation of a large set of constructs targeting genes overexpressed in ErbB-2 positive breast tumors. We have also developed a relatively rapid and reliable method for quantification of proliferation changes due to shRNA expression in cells. Using this we have identified approximately 30 genes that negatively impact the growth of Erb-B2 positive breast cancer cells.

Year 2 of funding resulted in tests of the specificity of the shRNAs' negative impact on the growth of Erb-B2 positive breast cancer cells. Effects of these shRNAs on normal cells and other breast cancer cell lines, identified approximately 20 shRNAs that *specifically* inhibit Erb-B2 positive breast cancer cells target genes. Several of these gene targets are known to be important to a variety of cancers. The PBP and NR1D1 genes are interesting because they give new insight into a pathway that can be exploited as a potential therapeutic target.

Year 3 of funding resulted in finding that inhibition of PBP-PPARγ-NR1D1 does not affect ERBB2 signaling in breast cancer cells. That this pathway is overactive in her-2/neu breast cancer cells is likely due to the tight genetic linkage of Nr1D1 and her-2. We have also found that the PBP-PPARγ-NR1D1 pathway alters fat metabolism in breast cancer cells and have identified lipotoxicity as a likely mechanism for triggering apoptosis in the her-2/neu positive breast cancer cells.

"So what?" one might ask.

Our unbiased approach has identified the PBP and NR1D1 genes, which are related to adipogenesis, as genes required by Erb-B2 positive breast cancer for survival. Using small molecule inhibitors and reporter experiments, we have developed a working hypothesis that adipogenic genes may be required for breast cancer proliferation. Further work has led to a working hypothesis in which amplification of genes from the 17q12-21 chromosomal region supercharge fatty acid production. ERBB2 upregulates FAS. PBP and NR1D1 function in conjunction with PPAR   to increase storage of triglycerides and allow for continued glycolysis. Inhibition of PBP, PPAR   or NR1D1 which positively influence several steps in fat production and storage, leads to palmitate accumulation and lipotoxicity resulting in apoptosis.

These genes and the pathway that they operate in are potential chemotherapeutic targets. Our ability to inhibit Erb-B2 positive breast cancer cell proliferation *in vitro* with a small molecule inhibitor of this pathway underscores the potential of this approach.

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